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Diploma Thesis

Preparation of cDNA of Selected Gene and Cloning

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Abstract

ABC drug efflux transporters are transmembrane proteins which are known to play a crucial role in drug resistance. The ability of these proteins to actively transport a wide variety of compounds out of the cell is one of the key reasons behind the resistance of cancer cells to chemotherapeutic agents. To study the activity of efflux transporters and identify their substrates or inhibitors a number of *in vitro* models have been developed. These models are based on the mammalian cells genetically modified in order to express the protein of interest. For genetic manipulations of the cells various expression plasmids are used. In our study we attempted to prepare a universal expression plasmid, containing a short sequence of V5 tag. Such plasmid enables further cloning of coding sequences of selected efflux transporters so that the V5 tag is localized on their 3'-end. Once constructed, the plasmid can further be used to transfect the desired cell line, thus developing an *in vitro* cell model. Since the transporter is produced as a fusion protein with the V5 tag localized on their C-end, the tag could be used as a universal marker of the transporter expression detectable by immunohistochemical methods. To construct the V5 tag sequence two approaches were employed both leading to the product of the desired sequence of V5 tag DNA. This sequence was further cloned into the selected plasmids: expression plasmid pZeoSV2(-) and retroviral plasmid pLNCX2. Only the cloning of V5 tag into pLNCX2 was successful in producing the universal expression plasmid pLNCXV5, prepared for insertion of cDNA of selected transporter.

ABC jsou velká skupina transmembránových proteinů, o kterých je známo že hrají klíčovou roli v rozvoji lékové rezistence. Schopnost těchto proteinů aktivně transportovat širokou škálu látek ven z buňky je hlavním principem – schopnosti řady nádorových buněk odolávat působení chemoterapeutik. Pro studium aktivity lékových transportérů a jejich substrátů či inhibitorů bylo vyvinuto množství *in vitro* modelů. Tyto modely jsou založeny na využití savčích buněk, které jsou geneticky manipulovány tak, aby v plazmatické membráně exprimovaly vybraný efluxní transportér. Pro genetické modifikace těchto buněk se využívá řada produkčních plazmidů. V naší práci jsme se zaměřili na vývoj univerzálního produkčního plazmidu obsahujícího krátkou V5 tag sekvenci a umožňujícího následné vklonování kódující sekvence vybraného lékového transportéru tak, aby byl V5 tag lokalizován na jeho 3' konci. Výsledný produkční plazmid tak může být dále použit k transfekci vhodné savčí buněčné linie, která bude sloužit jako model pro studium aktivity efluxního transportéru *in vitro*. Vybraný transportér je v buněčné linii produkován jako fúzní protein s V5 tagem lokalizovaným na jeho C-konci. Tak může tento tag sloužit jako univerzální marker proteinové exprese detekovatelný s pomocí imunohistochemických metod. Pro konstrukci V5 tagu jsme použili dva přístupy skládání oligonukleotidů. Oba tyto přístupy se nakonec ukázaly jako vhodné. Sekvence V5 tagu byla po izolaci vklonována do vybraných plazmidů: expresní plazmid pZeoSV2(-) a retrovirový plazmid pLNCX2. Klonování V5 tagu se podařilo pouze v případě pLNCX2. Vzniklý univerzální plazmid pLNCXV5 je tak připraven pro vklonování kódující sekvence vybraného lékového transportéru.

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I would like to thank Generi Biotech staff PharmDr. Zuzana Vackova Ph.D., Mgr. Vojtech Vejvoda Ph.D. and PharmDr. Antonin Libra PhD. for their help and guidance in completing this diploma thesis, as well as PharmDr. Miloslav Miletin Ph.D. for giving me this opportunity to work with Generi Biotech

1. Abbreviations:

ABC Transporters - ATP-binding cassette transporters

DNA - Deoxyribonucleic Acid

RNA – Ribonucleic Acid

mRNA – Messenger Ribonucleic Acid

CFTR - Cystic Fibrosis Transmembrane Receptor

BCRP – Breast Cancer Resistant Protein

MRP – Multidrug (Associated) Resistant Protein

TMD - Transmembrane Domains

NBD - Nucleotide Binding Domains

ICLs - Intracellular Loops

ATP - Adenosine triphosphate

ADP - Adenosine diphosphate

MDR- Multi Drug Resistance

TR(-) – Mutant Rat of MRP2

P-gp - P-glycoprotein

CF-1 mouse – P-glycoprotein mutant mouse

MDCK – Madin-Darcy canine kidney cell line

SV5 - simian virus 5

UV – Ultraviolet light

TBE - Tris-borate buffer

SOC medium - Super Optimal broth with Catabolite repression medium

PCR – Polymerase Chain Reaction

RT-PCR – Real Time Polymerase Chain Reaction

Pi - Phosphate

bp – base pair

LB - Lysogeny Broth

2. Introduction

ATP binding cassette transporters (ABC transporters) are a large group of transmembrane proteins that utilize energy of ATP hydrolysis to transport various substrates across the cell membrane, into or out of the cell. They transport ligands across the cellular lipid membranes, which are critical for most aspects of cell physiology including the uptake of nutrients, elimination of waste products, energy generation and cell signaling[1]. There are many different transporters, in both eukaryotes and prokaryotes, which are important for cells function and survival. ABC transporters are just one of these vast transporters crucial for cells survival. Their three most known functions are import, export and maintenance and repair of DNA[2]. However, in prokaryotes there are importers and exporters. The importers functions to transport nutrients into the cell across the membrane. These nutrients include sugars, amino acids, peptides, phosphate esters, inorganic phosphate, sulfate, phosphonates, metal cations, iron-chelator complexes, vitamins, and polyamines[3]. On the contrary, eukaryotes only have exporters[1]. Their function is to remove compounds such as drug or toxins that are found in the cell[1]. Some ABC transporters, located in the cytosol, are also involved in processes of maintaining and repairing DNA, and mRNA translation[2].

A large subgroup of ABC transporters called drug efflux transporters have recently been identified in mammals. These proteins act solely as effluxers and are able to transport a vast number of chemically distinct molecules out of the cell. They are expressed throughout the body especially in the plasma membrane of the intestine, kidney and liver epithelia and in physiological barriers (blood-brain, hemato-testicular, placental)[4]. In these tissues they affect absorption, distribution and elimination of their substrates, including drugs. Thus, modulating their therapeutic efficiency. Furthermore, the over-expression of some efflux transporters in cancer cells has been related to the phenomenon of multidrug resistance[5]. As these transporters can fundamentally affect drug efficacy, profound investigation of the potential interaction with efflux transporters is very important especially in the discovery of new therapeutic agents and their development.

3.1 ABC Transporters of Prokaryotes

Prokaryotic ABC transporters are responsible for the cells viability, virulence and pathogenicity[6]. There are many different ABC transporters in prokaryotes, each having its own special function and a role in the organism's survival. Hence, their dysfunction could lead to severe consequences leading to serious effects on the organism. Prokaryotes have both importers and exporters, which transport nutrients into the cell and toxins out of the cell for its viability[5]. They are predominantly unidirectional making their main function as importers of compounds that cannot be obtained by diffusion (sugars, vitamins, metal, ions, etc.) into the cell[6]. Other transporters are solely devoted to exporting virulence factors in suitable conditions. An example is iron ABC uptake systems, which have long been recognized as important effectors of virulence[6]. These pathogens obtain iron by releasing siderophores which have high affinity towards iron complexing molecules and once the iron siderophore complex is formed it is reabsorbed[6]. The iron complexing molecules, biologically available in the body, is found chelated by high-affinity iron-binding proteins (BPs) (e.g., transferrins, lactoferrins, and ferritins) or as a component of erythrocytes (such as heme, hemoglobin, or hemopexin)[6].

Another example of virulence is through the expression of virulence genes in *Agrobacterium tumefaciens*[7]. The virulence gene ChvE codes galactose and glucose importers. Once the sugar binds to regulatory region of ChvE gene it results in a response that expresses the virulence gene[7]. Prokaryotic ABC transporters are also responsible in maintaining homeostasis in the cell, which is very crucial in cells vitality[6]. An example of homeostasis is the osmosensing ABC transporters that mediate uptake of compatible solutes, which are activated once there is an increase in osmotic strength[6].

3.2 ABC Transporters of Eukaryotes

The main difference between eukaryotes and prokaryotes is that eukaryotes lack importers, therefore only have exporters. They are responsible for transporting toxins absorbed from the environment as well as metabolic by-products out of the cell[1]. Since they are one of many transmembrane proteins responsible for cells ability to function properly, their polymorphism may have a big impact on the organism. There are approximately 50 known ABC transporters in the human body. Currently, there are 13 known genetic diseases as a result of

their polymorphisms[8]. The most common genetic diseases include; cystic fibrosis, Stargardt disease, age related macular degeneration, adrenoleukodystrophy, Tangier disease, Dubin-Johnson syndrome and progressive familial intrahepatic cholestasis[8]. Certain ABC transporters responsible for these genetic disorders do not function by moving substrates across the membrane instead they control the ion channels[8], for example cystic fibrosis transmembrane receptor (CFTR). Polymorphism of ABCC7 (CFTR) leads to its dysfunction and acquirement of cystic fibrosis[8]. Summary of genetic disorders and the responsible transporters is seen in Table 1, below.

Table 1: Summary of human diseases associated with specific ABC transporters [8].This figure was obtained Gottesman MM and Ambudkar SV, 2001.

Disease	Transporter
Cystic Fibrosis	ABCC7 (CFTR)
Stargardt disease and age related macular degeneration	ABCA4 (ABCR)
Tangier disease and familial HDL deficiency	ABCA1 (ABC1)
Progressive familial intrahepatic cholestasis	ABCB1 (SPGP), ABCB4 (MDR2)
Dubin-Johnson syndrome	ABCC2 (MRP2)
Pseudoxanthoma elasticum	ABCC6 (MRP6)
Persistant hypoglycemia of infancy	ABCC8 (SUR1), ABCC9 (SUR2)
Sideroblastic anemia and ataxia	ABCB7 (ABC7)
Adrenoleukodystrophy	ABCD1 (ALD)
Sitosterolemia	ABCG5, ABCG8
Immune deficiency	ABCB2 (Tap1), ABCB3 (Tap2)

The normal function of some ABC transporters is to excrete cytotoxic compounds, such as dietary cytotoxics as well as therapeutic agents, from the cell. As mentioned previously the group so called drug efflux ABC transporters are associated with altered drug bioavailability and drug resistance. Among the most important drug efflux transporters in humans are: P-glycoprotein (P-gp, ABCB1, MDR1), Multidrug Resistance Associated Proteins 1-5 (MRP1-MRP5, ABCC1-ABCC5) and Breast Cancer Resistant Protein (BCRP, ABCG2)[4]. They are highly expressed in gut, liver and kidneys where they restrict the bioavailability of the administered drugs[4]. P-gp and BCRP are also expressed in the epithelia of sensitive tissue, for

example the brain, as well as in stem cells where they perform a barrier function[4]. Therefore, these transporters complicate the delivery process of therapeutic agents to the diseased cells. Another problem lies in the over-expression of ABC transporters in plasma membrane of cancer cells. Enhanced expression of the transporter results in enhanced efflux and decreased effective concentrations of the chemotherapeutic agent in the cell, leading to drug resistance. An example of this is the P-gp which is over-expressed in chemotherapy-resistant tumors, such as colon and kidney cancers, and is up-regulated after disease progression following chemotherapy in malignancies such as leukemia and breast cancer[9]. This topic will be covered further in detail later in the thesis.

On the contrary, expression of efflux transporters can also be beneficial, as is the case in pregnant women. Placenta plays a crucial role in delivering nutrients and exchanging waste between the fetus and the mother. There is evidence which demonstrates that the placenta expresses a large number of transporters, such as; P-gp, MRP1, MRP2, MRP3 and BCRP[10]. This indicates that ABC transporters, which cause drug resistance, may also serve as a protective mechanism of the fetus, preventing the transport of endogenous compounds and xenobiotics to the fetus[10]. Thus preventing any harm being done during its development.

4. Structure

To have a better understanding of function and mechanism of ABC transporters, we first need to have a good knowledge of their structure. In general all ABC transporters contain a core structure of two transmembrane domains (TMD) and two nucleotide binding domains (NBD)[11] (Figure 1).

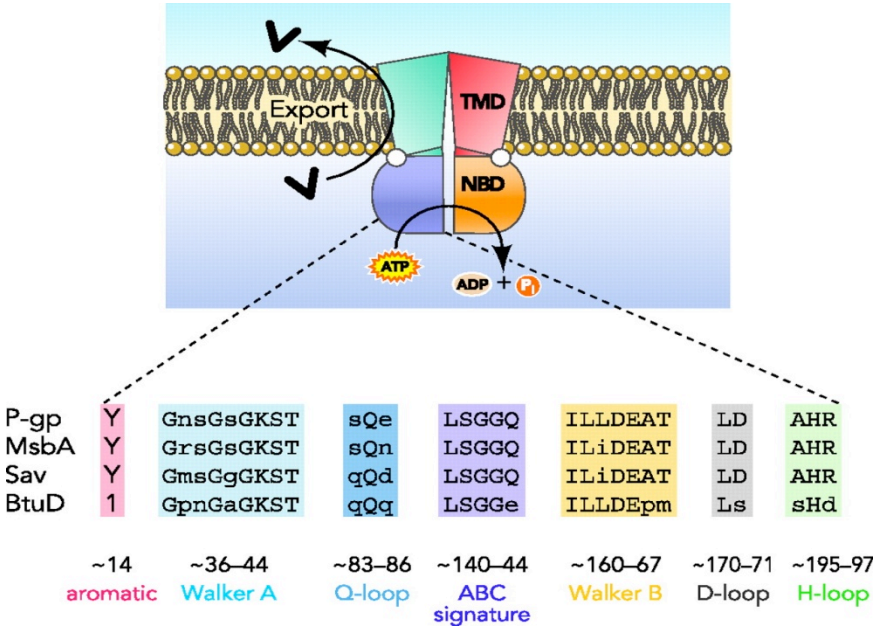


Figure 1: This is a general structure of an ABC transporter with two TMDs as well as two NBDs[1]. The two TMDs are located in the cell membrane with the NBDs attached to them[1]. This figure was obtained from Linton KJ, 2007.

The TMD is composed of 6-12 membrane spanning alpha helices and contain the specific substrate binding sites[11]. One of the most striking properties of ABC transporters is their ability to recognize and transport a vast variety of substrates of diverse chemical structure due to their variability in sequence and structure[11]. The two TMDs consist of hydrophobic segments, which span the membrane and form a transmembrane channel that allows nutrients or toxins to flow through[8]. While NBD are more organized with less variability in their structure. They contain characteristic motifs (Walker A and B), separated by approximately 90-120 amino acids, found in all ATP-binding proteins[11]. NBDs also contain an additional element, the signature (C) motif also known as LSGGQ motif, located just upstream of the Walker B[11], which is the diagnostic

sequence of ABC proteins[12] (Figure 2). Unlike TMD the NBD of ABC transporter is found in the cytosol where the ATP binding site is located[11].

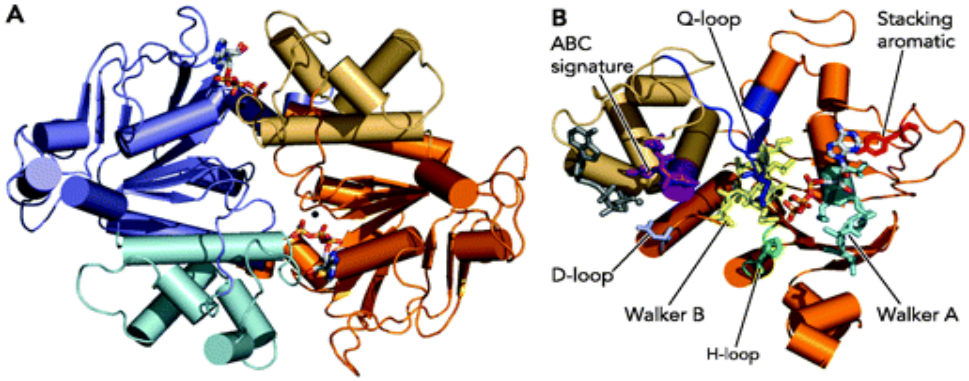


Figure 2: A picture of NBD dimer and conserved motifs[1]. Figure A: ABC transporter with closed NBD dimers (gold-blue and lighter gold-blue color), with two ATPs at the dimer interface[1]. Figure B: ATP is elementally colored and surrounded by Walker A and B motifs, the H-loop, stacking aromatic, and Q-loop of the core subdomain[1]. Q-loop extends from the top surface of the NBD, where it is close proximity to the ICLs of the TMDs, allowing the Q-loop to transduce signals and energy within the complex[1]. This figure was obtained from Linton KJ, 2007.

In order to become functional the transporters structure needs to be fully formed, consisting of four polypeptide chains, with the core structure being a C-terminal NBD connected to N-terminal TMD[12], together forming four component single polypeptide chain, NBD-TMD-NBD-TMD[12]. This is the usual sequence for the eukaryotic transporters found in for example P-gp. There are also exceptions to the polypeptide chain, with the most usual of these being half-transporters (eg. BCRP)[12]. However, these half transporters must also form either homodimers or heterodimers to form a fully functional transporter[11]. Another exception is the structure of some MRP transporter (MRP 1-3) where three instead of two TMD and two NBD pile up to form functional transmembrane protein, (refer to figure 3)[13].

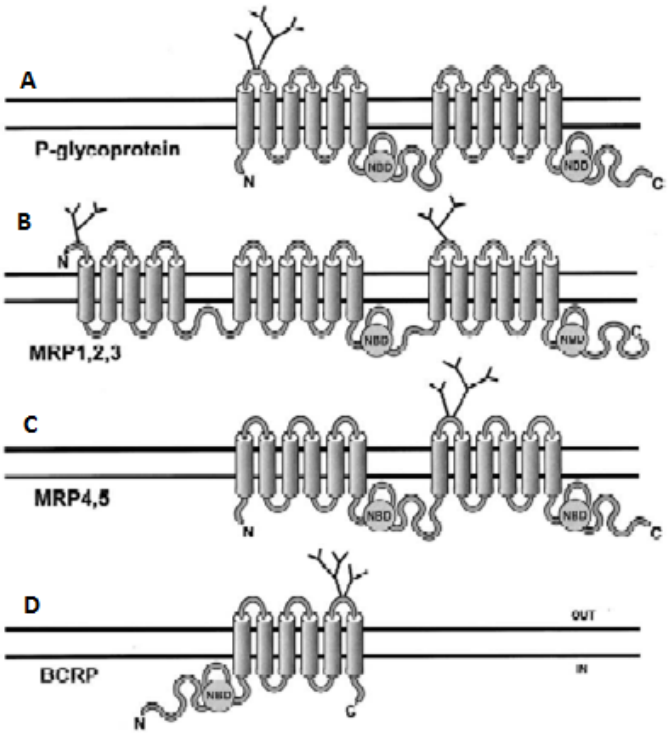


Figure 3: Comparison of alternate structures of ABC transporters[13]. Figure A: shows P-gp with two TMDs (with six transmembrane segments) and two NBDs, with the N-glycosylation on the first extracellular loop[13]. Figure B: MRP 1-3 have the same general structure as P-gp, but contain an additional aminoterminal extension with 5 transmembrane segments and are also *N*-glycosylated near the N-terminus and at the sixth extracellular loop[13]. Figure C: MRP 4-5 do not have the extra 5 transmembrane segment and are also *N*-glycosylated on the fourth extracellular loop[13]. Figure D: BCRP on the other hand is a half transporter containing only one NBD and a 6 transmembrane segments, with the N-glycosylation occurring on the third extracellular loop[13]. This figure was obtained from Schinkel A. and Jonker J., 2003.

From previous research we can see that the structure of ABC transporters, like the P-gp, has been examined through electron microscopy and electron cryomicroscopy showing that TMDs form an aqueous chamber[4]. The TMDs appear to be open at the extracellular face but closed intracellularly, with the two NBDs exposed as cytoplasmic lobes[4]. ABC transporters also contain the loops Q, D and H, which are found in the NBDs and play an important role in their function. The Q loop is a glutamine residue, which connects TMD and NBD, and is responsible for interaction between the two. The importance of the Q loop is due to its position in the transporter which allows it to couple the ligand binding sites within the TMDs to the ATP binding sites of the NBDs[1]. This allows a conformational change of TMDs during ATP

hydrolysis, resulting in translocation of the ligand (substrate). The H loop, which is the histidine residue, also contributes to the translocation through its interaction with ATP in the NBD[1].

5. Mechanism of Action

The four ABC transporter domains work explicitly together to translocate substrates through the cell membrane. This process involves communication via conformational changes, in both directions, between the NBDs and TMDs[4]. Since most of the focus is on the multi drug resistance proteins, the P-gp will serve to explain the mechanism of action. It is a four step mechanism, which cycles between high and low affinity states for ligands on different side of the membranes[1]. Additionally, it is known as the on-off switch where the NBDs conformational changes serve as the drive for transport[4]. NBDs closed dimer, one of the conformations, is formed when the two ATP molecules bind at its interface[4]. The other conformation, or the open dimer, results after ATP is hydrolyzed and ADP together with phosphate (Pi) are released allowing NBDs to open[4]. Each switch in the NBDs induces a conformational change in the TMDs, which is required for the translocation of the ligand[8]. For a better understanding refer to Figure 4 below.

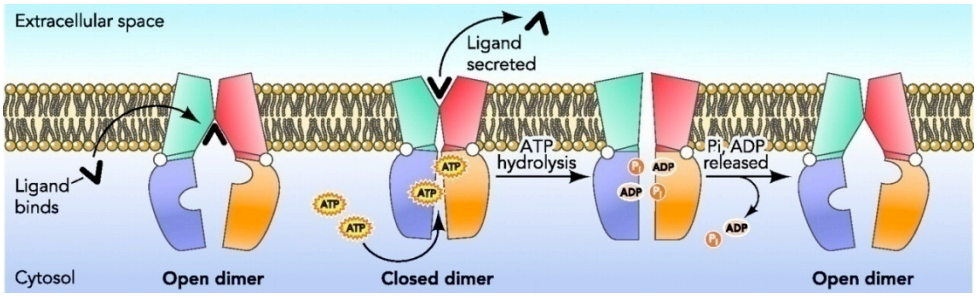


Figure 4: ABC transporters mechanism can be categorised into a four steps involved in the transportation of a substrate out of the cell. First the substrate binds to its high affinity site on TMDs, as a result conformational change occurs in the transporter. This change increases affinity towards ATP on the NBDs. Once ATP is bound it causes NBDs to close, which induce another conformational change in the TMDs. This allows TMDs substrate sites to change to low affinity and release the substrate. Once the ATP is hydrolysed and released in the form of ADP and Pi, the molecule returns to its resting state. This figure was obtained from Linton KJ, 2007.

First Step:

The ABC transporter is in basal state, meaning that the NBDs are in an open conformational state with a low affinity towards ATP, while the TMDs are in a high affinity state towards the ligands and are easily accessible from the cytoplasm of the cell membrane[1]. Once the ligand binds to its high affinity site on the TMDs it initiates the four step mechanism, or the translocation cycle, where the binding of the substrate initiates a conformational change in the

transporter[1]. The conformational change results in an increased affinity towards ATP on the NBDs[1]. The evidence to support this has been demonstrated from structural studies of HlyB and LolD that have concluded that the conformational change is transduced via intracellular loops (ICLs) of the TMDs, and the close proximity of ICLs to the Q loop and Walker A motif suggests that the binding of the substrate can directly influence the NBDs affinity towards ATP[1].

Second Step:

Once ATP has bound to its high affinity site on the NBDs, it causes the NBDs to change from opened dimer to a closed dimer around the bound ATP molecules[1]. This change in NBDs dimer induces a major conformational change in the TMDs to initiate substrate translocation[4]. The conformational adjustment allows the TMDs high affinity site, accessible in the cytoplasm, to change into low affinity site allowing the substrate to leave the transporter on the extracellular face of the membrane[1]. Because it is an active transporter, which requires energy to induce conformational changes, it was hypothesised that the energy produced by ATP hydrolysis was responsible for the conformational changes[1]. Recent research have concluded that ATP binding and formation of the NBDs closed dimer, and not the hydrolysis, generates a significant amount of energy to induce key conformational changes of the TMDs involved in substrate transport[1]. The extent of this conformational change is large and can be visualised directly for P-gp at a low resolution[1].

Third Step:

ATP is hydrolysed to initiate transition of NBDs from a closed dimer to an open dimer[1]. The hydrolysis destabilises the closed dimer to initiate the resettling of the transporter to its basal state[1]. The process of ATP hydrolysis initiation is still not fully known. However, two theories exist. The first theory postulates that once the substrate is released from the TMDs, it triggers a conformational change which is transmitted to the NBDs initiating the hydrolysis[1]. The second, more likely theory of the two, indicates that ATP hydrolysis is an automatic process upon the closure of the NBDs[1]. For certain ABC transporters like the P-gp, hydrolysis of both ATPs is required for completion of the transport cycle, and these two ATP molecules are hydrolysed at different times (non-simultaneously)[1]. While in other ABC transporters, like ABCC7 (CFTR),

hydrolysis of only one ATP molecule is sufficient to drive the transporter back through the translocation cycle[1].

Fourth Step:

The final step of the transport cycle is the release of Pi and ADP, restoring the transporter to its basal state. Once the Pi is released the ADP remains bound to the NBDs, however, it cannot maintain the stable state of the closed dimer resulting in ADPs release[1]. Again two theories exist of the mechanism behind the release of Pi and ADP, which are based on the analysis of the structure of HlyB (via exit channels) and LolD (following electrostatic repulsion of the two NBDs)[1]. The theory of exit channels suggests that these tunnels found in the NBDs are hydrophilic and contain a bound ATP molecule, which after hydrolysis is used for the release of Pi[1]. Since there are two NBDs with two ATPs bound to them, there are also two tunnels. One tunnel provides continuous passage from one ϒ-phosphate to the surface of the dimer, while the other tunnel extending from the ϒ-phosphate of the second ATP is closed by a salt bridge, explaining the non simultaneous hydrolysis of the two ATP molecules[1]. Therefore, it has been concluded that one Pi leaves the closed NBD dimer via exit tunnel without any build up of electrostatic charge[1]. The remaining ADP is unable to stabilise the NBD dimer, and the dissociation of the NBDs results after which the energy stored in the conformation is released to displace the ADP[1]. The second theory that has been proposed is that the electrostatic repulsion between the ADP coordinated by the core sub-domain of one NBD and the Pi coordinated by the signature motif of the other NBD destabilises the closed NBD dimer, leading to Pi and ADP release[1]. Once this occurs the transporter is restored to its basal state.

This four step process of ligand translocation gives us a general idea of how this mechanism works. There might have been some minor details that were omitted, but this process allows us to have better understanding of function of the ABC transporters.

6. Relevance to Drug Resistance:

For some time now, ABC transporters have been known for their role in the development of resistance to therapeutic agents, which is a very common problem in infectious diseases as well as in cancer. Cells recognize medication as a toxic compound, and in order to protect the organism from the toxic substance they expel it out of the cell which may eventually lead to resistance. Patients who are receiving a therapeutic agent may develop resistance to that agent, and as a result also may develop resistance to several other therapeutic agents[5]. The way that cells develop this resistance can be acquired in several different ways some of which include: decreased uptake, increased detoxification, alteration of target proteins, or increased excretion[12]. Over-expression of the protein results in the increased excretion, which is the key behind drug efflux transporter mediated resistance[5]. Hence, this has been the main concern in humans regarding resistance to chemotherapeutic agents[5]. However, it is also important in eukaryotic as well as prokaryotic resistant microorganism[5]. There are a number of efflux transporters in leukemia and in tumour that are responsible for resistance, however, there are only three main proteins which are responsible for almost all the resistance occurring in neoplastic cells[14], which include P-gp, MRP1 and BCRP[4]. There are five more known ABC transporters which are also considered to be responsible for resistance in cancer cells; MRP2, MRP3, MRP4, MRP5 and MRP6[14]. In general P-gp and BCRP preferentially extrude large hydrophobic, positively charged molecules, while the members of the MRP family can extrude both hydrophobic uncharged molecules and water-soluble anionic compounds[5]. Some of the examples of substrates which are transported by these three main transporter proteins are seen in Table 2.

Table 2: Specific Substrates of P-gp[11], MRP[11, 15] and BCRP [11, 16]

ABC Transporter	Specific Substrates
P-gp	Colchicine, etoposide, quinidine, vinblastin, tacrolimus, adriamycin, bilirubin, peptides, lipids, steroids,protease inhibitors, xenobiotics, cardiac glycosides, glucocorticoids, non-nucleoside reverse transcriptase inhibitors
MRP 1	Doxorubicin, daunorubicin, vincristine, colchicines, VP-16, etoposide, rhodamine, *S*-nitrosoglutathione, 17β-estradiol-17β-D-glucuronide
BCRP	Anthracycline, topotecan, mitoxantrone, doxorubicin, CPT-11, daunorubicin, rhodamine, prazocin, methotrexate, flavopiridol, idarubicinol, epirubicin, topotecan

For a better understanding of the mechanism behind ABC transporters influence in resistance, we shall examine the P-gp function in more detail. P-gp is able to cause the highest resistance to bulky amphipathic drugs, such as paclitaxel (taxol) and anthracyclines[17]. The hydrophobic parts of these drugs allow their rapid insertion into the membrane[17]. The hydrophilic residues prevent rapid flipping of the drug to the inner leaflet of the membrane, slowing down entry into the cell; in fact, for an anthracycline like doxorubicin (Adriamycin) this takes about a minute, giving the P-gp pump plenty of time to deal with the influx of doxorubicin[17].

Extensive research has been conducted in regards to overcoming resistance and different methods have been postulated in which chemotherapy could be improved. One very prominent method is the inhibition of efflux transporters which are responsible for the multi drug resistance. In order for this method to work, these inhibitors must be highly specific to the efflux transporters present in the neoplastic cells. An example of a potent inhibitor of transport function is vanadate, which is able to stabilize NBD dimer with one ATP molecule and one ADP molecule. This occurs during the third stage of the transport cycle after ATP hydrolysis, once the Pi is released and exits the NBD dimer it can be replaced by vanadate causing the transporter to be shunted into a stable conformation[4, 18]. Biochemical evidence suggests that it is an activated state with a distinct conformation of the TMDs of P-gp, which remains with a low affinity towards vinblastin[4,18]. Inhibitors such as vanadate have the ability to treat drug resistance in cancer, including eukaryotic as well as prokaryotic resistant microorganisms[4].

The investigation of the interactions between drug efflux transporters and their substrates or inhibitors is through the development of various *in vitro* and *in vivo* experimental models. These models can be used for detection of new therapeutic agents that interact with drug efflux transporters. Thus specifically identifying the agents to which the neoplastic cells may be resistant towards, prior to their development and introduction to the market. Furthermore, they can also help to identify the agents which cause inhibition of ABC transporters and can be potentially used to enhance drug bioavailability and efficacy.

7. *In vitro* and *in vivo* models of drug efflux transporters

Since the discovery of drug efflux transporters and the role they partake in the impairment of efficacy of therapeutic agents, scientists have been searching for a solution to resolve this problem. This research has taken us towards the development of numerous *in vitro* and *in vivo* models of ABC transporters that allow the discovery of interactions between efflux transporters and their potential substrates or inhibitors. Different models allow the focus on the activity of transporters including the early identification of therapeutic agent, kinetic parameters, directionality of transport and tissue specificity of a particular drug efflux transporter[19].

7.1 *In vitro* Models

Different types of *in vitro* methods are used to examine the activity of efflux transporters, and the selection of each method depends on the desired output[19]. *In vitro* assays might be divided into three main categories: accumulation/efflux assays, transport assays and ATPase activity assays[19]. Both parental and transfected cells over-expressing a particular drug efflux transporter are employed.

Initially accumulation and efflux studies will be discussed, which can be done using cell suspension, cell monolayer, or membrane vesicles[19]. In these studies, the translocation of selected compound into or out of the cells across the plasmatic membrane is investigated. The most common method to trace the localization of tested substrate is by using a probe which is typically either fluorescent or radiolabeled. The final location of these probes indicates whether the substrate movement across the cell membrane is influenced by the efflux transporter[19]. Accumulation studies examine the movement of the probes into the cells or membrane vesicles under controlled experimental conditions, and in the presence or absence of specific inhibitors of the drug efflux transporter[19]. If the tested compound is a substrate of the efflux transporter, the addition of the inhibitor will result in an increase of the probe amount in the cells. During efflux studies, cells are pre-loaded with the probe of interest and the resulting transport of the probe out into the extracellular environment is measured under various conditions known to influence transporter activity[19,20]. One of the main outcomes of these studies is the detection of new potential therapeutic agents which inhibit the transporters activity. Hence, the accumulation/efflux method can be used in early stages of drug research. Therapeutic agents showing initial activity in the first screening assay can then be subjected to further investigation,

using additional *in vitro* or *in vivo* functional assays to provide more detailed information about actual activity of the compounds[19].

Second group of *in vitro* methods are the transport assays, which has the same function as accumulation/efflux assay that is to identify the agent's specificity to the efflux transporters. This is accomplished by using confluent cell monolayer cultured on a permeable membrane support matrix[19]. A variety of cells may be used for this assay, however, they must express the drug efflux transporter of interest, and form a functional polarized cell monolayer together with tight extracellular junctions[19]. In order to express the transporter of interest, these cells are transfected with a plasmid carrying the coding sequence of a particular efflux transporter. According to its type the transporter, they can be localized either on the apical or basolateral side of the cell monolayer thus affecting the transport of the substrate in different directions. Successfully transfected cells are then used to identify the agents which are specific to the individual transporter[19]. Once the assay is ready, the fluorescent or radiolabeled agent is placed on the cell monolayer in three possible ways: on the apical side, on the basolateral side or equal concentrations on both the apical and basolateral sides (known as equilibrium transport assay)[19]. In order to determine whether the tested compound is a substrate of the particular efflux transporter, the flux of the tested compound across the confluent cell monolayer must be measured. In the case of application of substrate on the apical or basolateral side, the activity of the transporter can be determined through bidirectional difference in compound permeability which can be influenced by inhibitors[19]. In the equilibrium transport assay, the distribution of the agent, up to 48 hours, is followed by expressing the apical to basolateral ratio of the agent allowing the activity of the efflux transporter to be obtained[19]. Transport assays can be used to determine the effect of drug efflux transporters on the permeability of the agents, as well as to help identify cellular localization of the drug efflux transporters in plasma membrane[19,21].

Finally, the last *in vitro* methods are ATPase assays. As it can be seen from its name it incorporates the ATPase activity of the transporters, monitoring the hydrolysis of ATP. Since all ABC transporters require ATP to move their substrates across the cell membrane, hydrolysis will indicate which substrate is specific to the selected drug efflux transporter[19,22]. Components required for this ATPase assay are: cell membrane preparation enriched with the transport protein of interest, ATP, analytical method for the detection of inorganic phosphates generated from ATP hydrolysis, and a mechanism discriminating between general ATPase activity and

ATPase activity related to the transport of interest[19]. The ATPase activity of the specific transporter in question is distinguished from other ATPase activity through the inclusion of various inhibitors such as ouabain or azide which inhibit Ca-ATPase, Na/K-ATPase and mitochondrial ATPase respectively[19]. The ATPase assay is also used to identify substrates specificity of the transporters, as well as to identify the inhibitors. ATPase assays have been used most extensively in examining P-gp drug efflux transporter[19]. ATPase assays can also be used to examine the changes in basal ATPase activity to quantitatively determine potential differences in the affinity of compounds for drug efflux transporters[19,23].

7.2 *In vivo* Models

In vivo models examine the activity of drug efflux transporters slightly different then *in vitro* models. *In vitro* models are used to determine interaction between transporters and their substrates as opposed to *in vivo* models, which examine the influence of the transporter on absorption, distribution and elimination of the therapeutic agents in the organism[19]. Transgenic and mutant animals are the most common *in vivo* models used[19].

Transgenic models are genetically modified animals where the transporter of interest can be either deleted or overexpressed[19]. The way these transporters are deleted or expressed is done through a process called gene knockout or homologous recombination[19]. In this process, the targeting vector carrying the DNA sequences which are homologous to the genes of interest are introduced into an embryonic stem cell[19]. The insertion of the sequence into the stem cell causes the target gene to be inactive producing desired embryonic stem cells that express this inactive gene. Once it is identified, it is injected into the blastocysts and is implanted into the foster mothers[19]. The offspring are examined for the disrupted alleles and inbred to produce a stable line of animals which are homozygous for the deleted gene[19]. The usual selected animal is mice[19]. P-gp and MRP-1 have been the two most widely used efflux transporters employed in the transgenic models. When examining P-gp a slight difference in the human and mice genome needs to be acknowledged, as mice have two genes encoding the P-gp *mdr1a* and *mdr1b,* while humans have only one gene (MDR1) encoding this transporter[19]. Hence, in order to examine P-gp function in drug pharmacokinetics and efficacy, one or both of these genes need to be knocked out[19]. This has led to a number of studies for a better understanding of P-gp function, such as its role in maintaining the integrity of the blood brain barrier, but most importantly its

understanding in disposition and elimination of different substrates[19]. The significance of these studies is the substrate, which may be identified using *in vitro* efflux transporters models and on the other hand may not be identified as a substrate in more complex animal models such as the transgenic model. Hence, allowing the transgenic model to confirm whether the transporter actually affects the substrate kinetics in live animals[19].

The second *in vivo* model on the other hands is composed of two mutant animals, which are not genetically modified, but are rather naturally mutated to lack the transporter proteins that are responsible for resistance. The first mutant model is a sub-population of the CF-1 mouse that exhibits a genetic defect, resulting in the absence of P-gp expression[19]. This genetic defect occurs in *mdr1a* gene where an insertion of DNA occurs at exon 23, resulting in aberrant splicing of the mRNA and the loss of exon 23 during RNA processing[19]. The sub-family has been bred to produce off-springs that display this phenotype for further evaluation of P-gp function in pharmacokinetics, disposition and toxicity[19]. The second mutant model is known as TR(-) rat, which lacks the gene for *mrp2*[19]. It is described as autosomal recessive conjugated hyperbilirubinemia, which displays impaired biliary excretion of conjugated bilirubin due to the naturally occurring deficiency in *mrp2* gene[19]. This mutant model has been primarily used as an animal model for Dubin-Johnson syndrome, since the phenotype of the TR(-) mutants is similar to the Dubin-Johnson syndrome in humans[19]. Additionally, it is used to study the absorption, distribution, metabolism and excretion of substrates, and elimination of their metabolites[19]. For example, the biliary elimination of acetaminophen glucuronide, where more than a 300-fold decreased in biliary excretion of acetaminophen glucuronide is observed in TR(-) rats compared to wild type controls[19]. Further research has also resulted in observing lower biliary excretion of both parent molecules and metabolite of in anticancer agents[19]. Both of these models are important in the study of efflux transporter protein activity in a living organism, giving us further clues as to how to treat different diseases as well as overcome transporter mediated multidrug resistance.

Since the mice genes vary from the humans genes, there are different considerations we need to for take when concluding the results derived from these animal studies. As explained before, the P-gp of the mice has two genes the *mdr1a* and *mdr1b* which induce drug resistance, thus silencing only one of these two genes might not be sufficient in fully suppressing the P-gp expression[19]. Certain organs of the mice might express one of the two genes or both, therefore

depending on the organ that is being studied they can still remain functional despite the removal of one of the genes[19]. Additionally, due to organism natural ability to compensate for certain defects such as in the case of a missing gene, the organism has the ability to overcome this by expressing other genes related to the missing one, such is the case for P-gp *mdr1b* gene[19]. When the *mdr1a* gene is genetically removed, there is an up-regulation of the *mdr1b* gene in the kidney and liver[19]. However, if the *mdr1b* gene is genetically removed there is no up-regulation of the *mdr1a* gene[19]. This removal of the genes can lead to undesirable outcomes such as altered pharmacokinetics, lack of viability or system toxicity, since it affects other organs than just the one of interest[19]. Hence, it would be ideal to develop a model which would knockout the genes of interest but also selective to the specific organs[19]. When comparing the human MDR1 (responsible for human P-gp) to *mdr1a* and *mdr1b* (mice P-gp) expressing cells, they demonstrate species dependent substrate specificity of P-gp[19]. Although majority of compounds which are indentified as P-gp substrates in the mutant CF-1 model and the transgenic *mdr1a* over-expressing model are also likely to be substrates for human P-gp, there is a great potential for either under- or over-estimation of drug susceptibility to P-gp in humans[19]. Here, the combination of *in vitro* and *in vivo* models is developed to overcome this problem[19]. The human *in vitro* assays are used to form and compare the results to those observed in the mice *in vivo* models to identify differences in P-gp substrate specificity of the two species[19].

8. Aim of Study:

The goal of this study was to prepare a universal plasmid (pZSV5 or pLNCXV5), which contains a short sequence of V5 tag. The V5 epitope tag is derived from a small epitope (Pk) present on the P and V proteins of the paramyxovirus of simian virus 5 (SV5). The V5 tag is usually used with all 14 amino acids (GKPIPNPLLGLDST), although it has also been used with a shorter 9 amino acid sequence (IPNPLLGLD). The V5 tag functions as a universal marker of protein expression, which could be detected by immunoanalytical methods (e.g. Western blot analysis). The newly prepared pZSV5 or pLNCXV5 plasmid enables the cloning of coding sequences of selected drug efflux transporters enabling the localization of V5 tag on their C-end. The final construct carrying the coding sequence of the desired efflux transporter can be then used for production of stable transfected cell lines which will serve as an *in vitro* model for investigation of drug efflux transporters.

9. Materials and Methods

9.1.1 Materials

All restriction enzymes were commercial obtained from New England Biolabs (Ipswich, Massachusetts, USA). The DNA markers used for gel electrophoresis were obtained from Fermentas (St. Leon-Rot, Germany). pZeoSV2(-) plasmid was purchased from Invitrogen Life Technologies (Carlsbad, California, USA). pLNCX2 retroviral plasmid was purchased from Clontech (Mountain View, California, USA). All oligonucleotides used for V5 tag were produced at Generi Biotech (Hradec Kralove, Czech Republic). RecoChip used for isolation of DNA from agarose gel after gel electrophoresis was obtained from Takara (Madison, Wisconsin, USA).

9.1.2 Equipment

For cell culture Binder Incubator was used (Vienna, Austria). Incubator with mixing capability of 220 rpm was from New Brunswick Scientific (Edison, New Jersey, USA). For sample centrifugation, different centrifuges were used: Sigma Laboratory Centrifuge 4K15C (Osterode am Harz, Germany), Eppendorf minispin centrifuge (Horsholm, Denmark) and Scan Speed 1730R centrifuge (Narellan, Australia). For brief mixing bio vortex from Biosan (Warren, Illinois, USA) was employed. Gel electrophoresis was performed using Hoefer HESS Mini Horizontal Submaine Unit and Bio-Rad power pack (Hercules, California, USA). Visualization and analysis of the gel was accomplished in G-box from Syngene (Cambridge, Great Britain) using the Gene Snap software. Concentration of DNA was measured using Spectrophotometer Nanodrop ND-1000 (Wilmington, North Carolina, USA). Transformation and isolation of *E.*coli was carried out in Biohazard box Aura 2000 BS (Pavia, Italy). Samples were heated in Omni Gene Heater (Kent City, Michigan, USA). For pipetting automatic pipettes from Eppendorf (Horsholm, Denmark) were used.

9.2 Cloning of pZSV5 and pLNCXV5 plasmids

9.2.1 Cloning Scheme of pZSV5

The pZSV5 plasmid is a modified commercial pZeoSV2(-) plasmid obtained from Invitrogen Life Technologies (Figure 5).

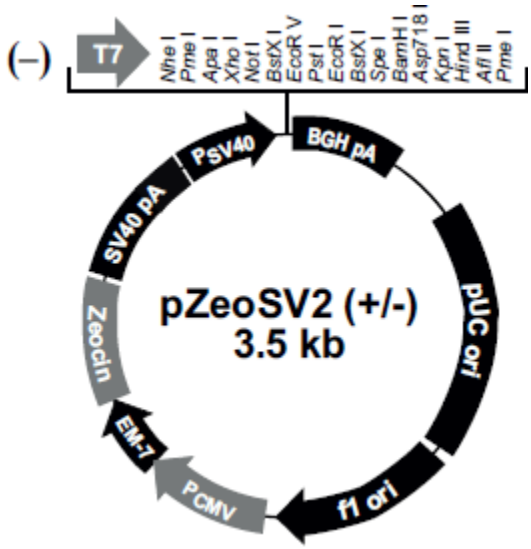


Figure 5: pZeoSV2(-) plasmid map. This figure was obtained from Invitrogen life technologies lab manual[24]

pZeoSV2(-) is a 3.45 kb vector which contains antibiotic (zeocin) resistance gene, allowing selection in both prokaryotes and eukaryotes[24]. This vector contains multiple cloning site, which enables the insertion of desired DNA sequences of interest (in our case the ABC transporter as well as the V5 tag). The expression of cloned DNA in mammalian cells is driven by potent eukaryotic P_{SV40} promoter.

At first the artificial sequence of the V5 tag has been designed so that it codes for the 14 amino acids (GKPIPNPLLGLDSTGTHHHHHH) of the natural V5 tag, six amino acids of 6-His tag (6xHis) separated from V5 tag by glycine (G) and threonine (T), and TGA stop codon at the end (Figure 6).

G K P I P N P L L G L D S T G T H H H H H H *
GGC AAG CCC ATC CCC AAC CCC CTG CTG GGC CTG GAC AGC ACC GGT ACC CAT CAT CAC CAT CAC CAT TGA GTA AAT AG

Figure 6: Amino acid and nucleotide sequences of the V5 tag

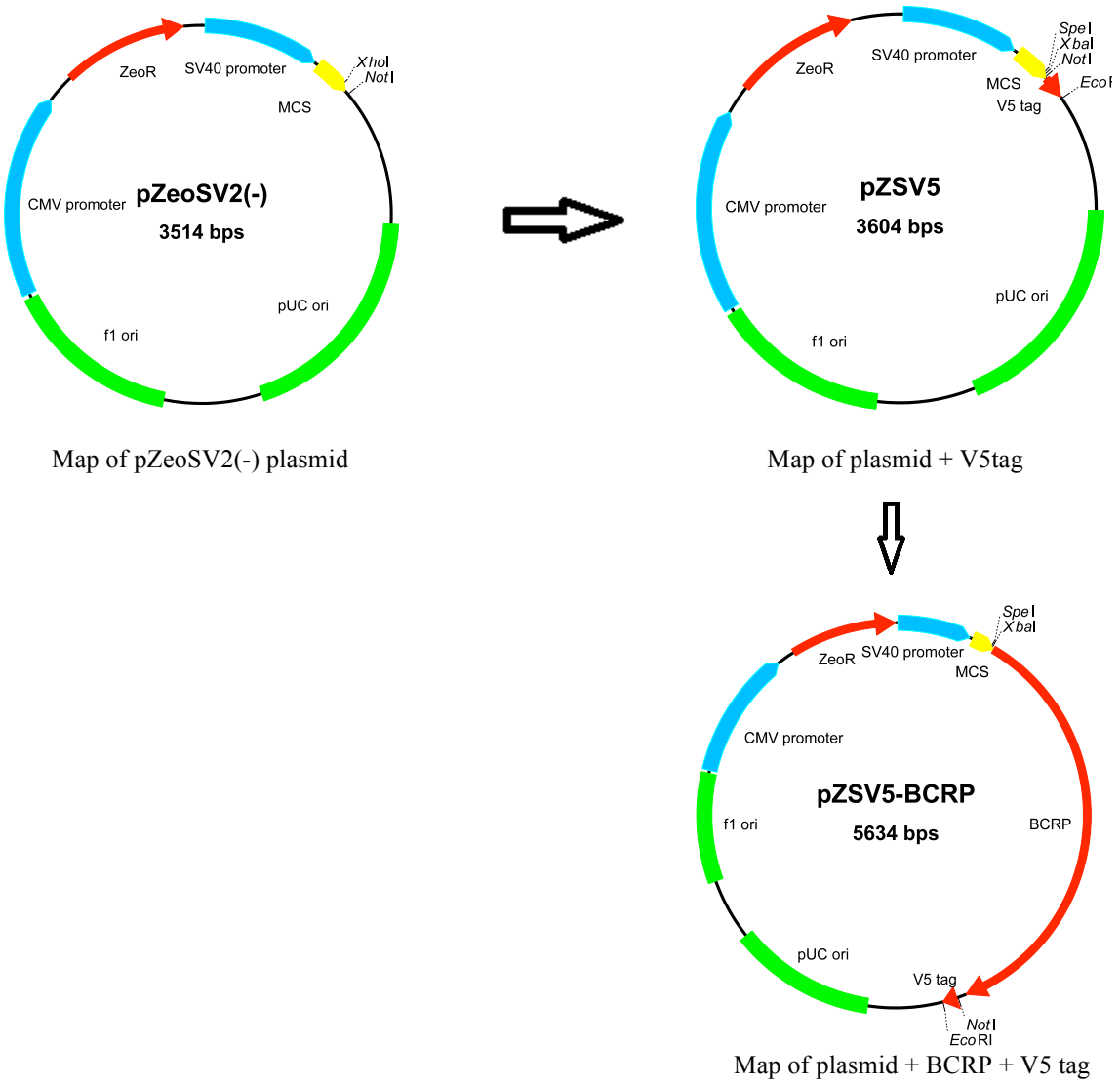
To enable cloning of the V5 tag DNA into the pZeoSV2(-) cohesive ends enabling the insertion of the sequence into *XhoI* and *NotI* restriction sites have been added to 5'- and 3'-end of the artificial sequence, respectively. Cohesive ends have been designed so that the restriction sites were annihilated after successful ligation. Furthermore, recognition sites for *SpeI, XbaI, NotI* and *EcoRI* were included into the sequence to allow for cloning of coding sequences of desired efflux transporters in frame with artificial V5 tag located on their C-end (table 3 lists different restriction endonucleases used for cloning of different transporters into pZeoSV2-). The resulting length of the V5 tag is 96 bp (Figure 7). For detailed scheme of the cloning procedure refer to Figure 8.

Table 3: List of restriction endonucleases with their recognition sites

XhoI	C|TCGAG GAGCT|C
SpeI	A|CTAGT TGATC|A
NotI	GC|GGCCGC CGCCGG|CG
XbaI	T|CTAGA AGATC|T
EcoRI	G|AATTC CTTAA|G

TCGACTAGTTCTAGAGCGGCCGCGGCAAGCCCATCCCCAACCCCCTGCTGGGCCTGGACAGCACCGGTACCCATCATCACCATCACCATTGAATTC
GATCAAGATCTCGCCGGCGCCGTTCGGGTAGGGGTTGGGGGACGACCCGGACCTGTCGTGGCCATGGGTAGTAGTGGTAGTGGTAACTTAAGCCGG

Figure 7: Double Stranded Sequence of V5 tag



Map of pZeoSV2(-) plasmid

Map of plasmid + V5tag

Map of plasmid + BCRP + V5 tag

Figure 8: Cloning scheme of BCRP into pZSV5.
To clone the DNA of interest (V5 tag and ABC transporter) into pZeoSV2(-), the plasmid is first digested either by *XhoI* or *NotI* restriction endonucleases. The sequence of V5 tag is inserted via ligation with the linearized plasmid, forming the new pZSV5 plasmid. This plasmid can be further digested using *SpeI/XbaI* and *NotI* restriction endonucleases, to enable the insertion of the cDNA of selected efflux transporter in frame with the V5 tag sequence, so that the resulting protein is produced with the V5 tag on its C-end. In our case the selected transporter was BCRP.

9.2.2 Cloning Scheme of pLCXV5

pLNCX2 is a retroviral plasmid which was also used to ligate with the V5 tag, as an alternative to the pZeoSV2(-) plasmid. pLNCX2 is a ~ 6.1 kb retroviral vector which contains antibiotic (neomycin) resistance gene, allowing selection in eukaryotes[25]. This vector contains multiple cloning site, which enable insertion of desired DNA sequences of interest (in our case

the ABC transporter as well as the V5 tag), seen in figure 9. The expression of the desired cDNA is eukaryotic cells is driven by potent $P_{CMV\ IE}$ promoter.

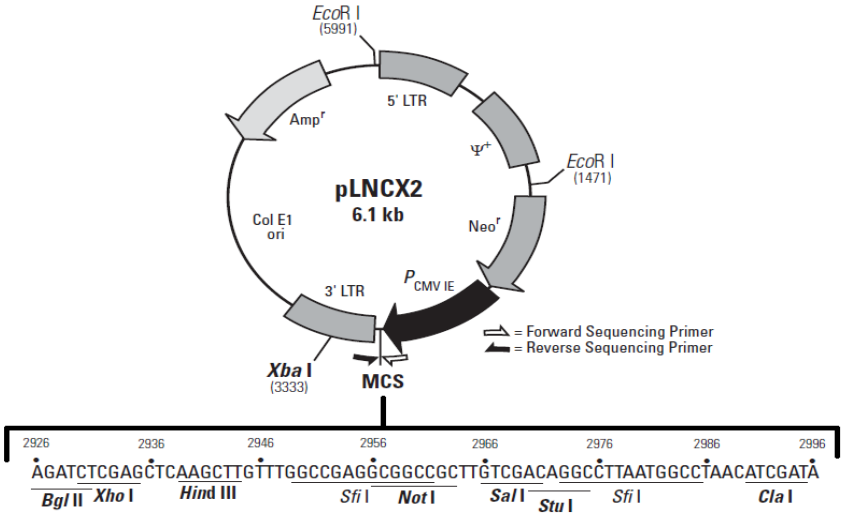


Figure 9: pLNCX2 plasmid map[25]. This figure was obtained from Clontech lab manual

The V5 tag DNA used to clone into pLNCX2 retroviral plasmid was the same one used to clone into pZeoSV2(-) plasmid (figure 5). In order to clone the V5 tag DNA into the pLNCX2 retroviral plasmid restriction endonucleases *XhoI* and *NotI* were used to digest and linearize the plasmid. Restriction endonucleases s *SpeI/XbaI* and *NotI* can further be used to clone the coding sequence of desired efflux transporter into the pLNCXV5 plasmid so that the resulting proteins are produced with the artificial V5 tag located on their C-end (table 4 lists different restriction endonucleases s used for cloning of different transporters into pLNCX2). For detailed scheme of the cloning procedure refer to Figure 10.

Table 4: List of restriction endonucleases with their recognition sites

XhoI	C|TCGAG GAGCT|C
SpeI	A|CTAGT TGATC|A
NotI	GC|GGCCGC CGCCGG|CG
XbaI	T|CTAGA AGATC|T

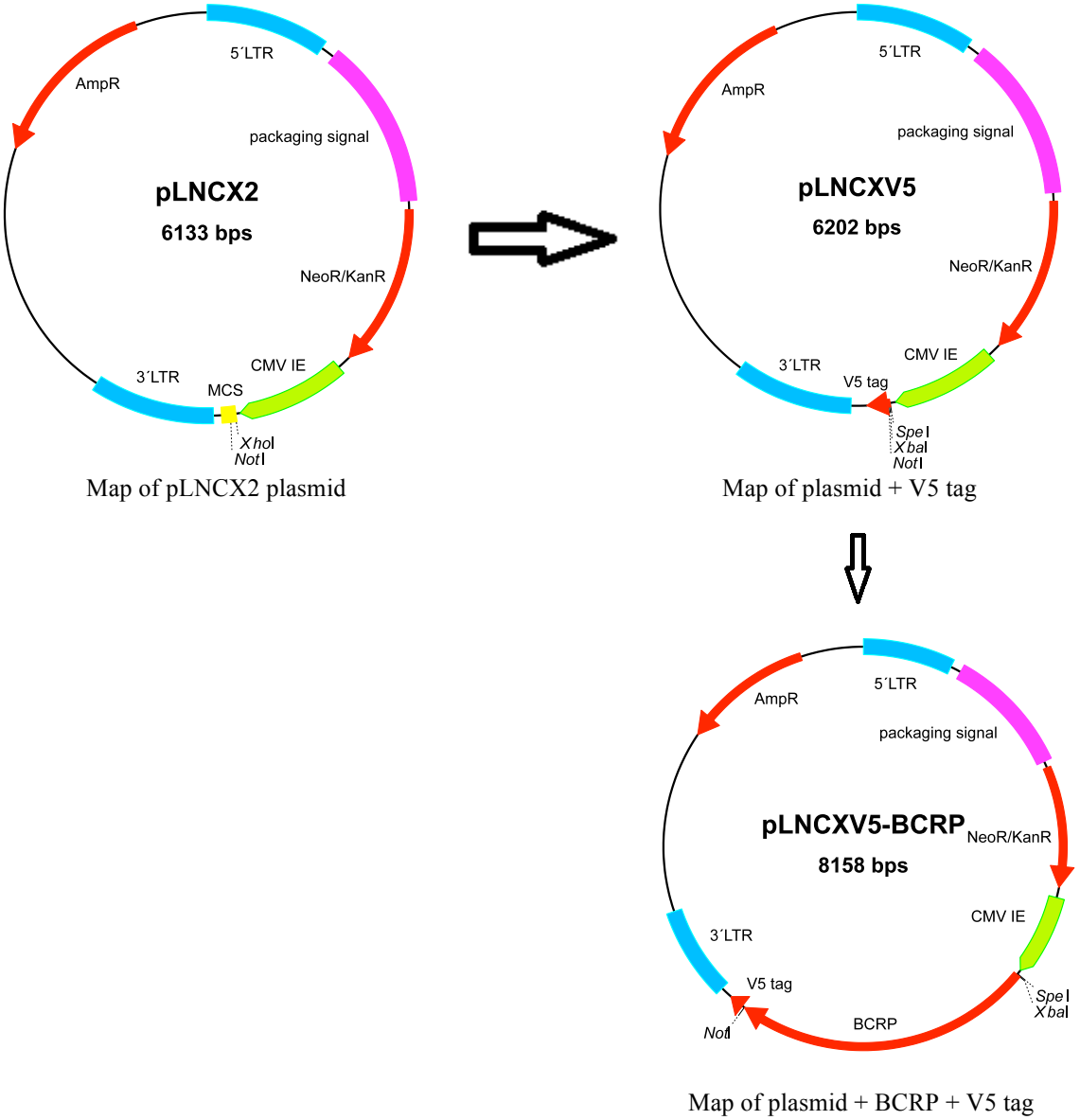


Figure 10: Cloning scheme of BCRP into pLNCX2
To clone the DNA of interest (V5 tag and ABC transporter) into pLNCX2, the plasmid was first digested using *XhoI* and *NotI* restriction endonucleases. The sequence of V5 tag was inserted via ligation with the linearized plasmid, forming the new pLNCXV5 plasmid. This plasmid was further digested using *XbaI and NotI* restriction endonucleases, to enable the insertion of the cDNA of selected efflux transporter in frame with the V5 tag sequence. In our case the selected transporter was BCRP. Thus, the final plasmid enables the expression of BCRP transporter with V5 tag sequence at its C-end.

9.2.3 Preparation of V5 tag

The artificial double stranded DNA sequence of the V5 tag has been assembled either from hybridization and subsequent ligation of four short sequences of oligonucleotides (V5N1, V5N1r, V5N2 and V5N2r, refer to Figure 11) or from simple hybridization of two oligonucleotides (V5_F and V5_R, refer to Figure 12).

V5N1: TCGACTAGTTCTAGAGCGGCCGCGGCAAGCCCATCCCCAACC
V5N1r: GTCCAGGCCCAGCAGGGGGTTGGGGATGGGCTTGCCGCGGCCGCTCTAGAACTAG
V5N2: CCCTGCTGGGCCTGGACAGCACCGGTACCCATCATCACCATCACCATTGAATTC
V5N2r: GGCCGAATTCAATGGTGATGGTGATGATGGGTACCGGTGCT

Figure 11: Short V5 tag oligonucleotide sequences

V5_F: TCGACTAGTTCTAGAGCGGCCGCGGCAAGCCCATCCCCAACCCCCTGCTGGGCCTGGACAGCACCGGTACCCATCATC
V5_R: GGCCGAATTCAATGGTGATGGTGATGATGGGTACCGGTGCTGTCCAGGCCCAGCAGGGGGTTGGGGATGGGCTTGCCG

Figure 12: Long V5 tag oligonucleotide sequences

Different temperature and concentration profiles have been used to optimize the hybridization of oligonucleotides. The first temperature profile involved incubating Mixtures A (V5N1+V5N1r) and B (V5N2+V5N2r) at 80°C for 2 minutes. The second temperature profile was completed in such a way that Mixtures A (V5N1+V5N1r) and B (V5N2+V5N2r) were heated for 3 minutes at 90°C, followed by a 10 minutes long decrease in temperature to 70°C, then heated for 3 minutes at 70°C, followed by slow 10 minute temperature decrease to 37°C and finally were heated for 3 minutes at 37°C. The third optimization profile involved incubating mixtures of two different concentrations; Mixture 1A (V5N1+V5N1r) and 1B (V5N2+V5N2r) were diluted ten times while the second Mixture 2A (V5N1+V5N1r) and 2B (V5N2+V5N2r) were mixed using the original stock concentration. Mixtures 1A, 1B, 2A and 2B were incubated at 95°C for 3 minutes and further incubated at room temperature over night. During this process denaturation and hybridization of complementary sequences of oligonucleotides occurred.

For hybridization of cohesive ends of hybridized oligonucleotides the A and B mixtures were mixed together and heated for 5 minutes at either 40°C or 95°C, then allowed to cool at room temperature for 20 to 60 minutes, respectively.

To hybridize the oligonucleotides V5_F and V5_R two starting concentrations were tested: 10-times diluted mixture 3 (V5_F+V5_R) and undiluted mixture 4 (V5_F+ V5_R). Both samples were incubated at 95°C for 3 minutes, and then allowed to cool at room temperature over night.

To complete the assembly of the V5 tag DNA from the oligonucleotides (V5N1, V5N1r, V5N2 and V5N2r) the final step of the process was ligation. Once the hybridization of cohesive ends was complete T4 DNA Ligase and T4 DNA Ligase buffer were added to the mixture, and incubated at room temperature for 20 to 60 minutes. In this final step ligation of the DNA fragments occurs, producing the desired artificial V5 tag DNA.

In order to determine whether the V5 tag DNA was successfully created and to isolate the sequence, gel electrophoresis method was employed. The V5 tag DNA was separated on agarose gel (2.0% or 2.5%). The gel used was submerged in 1xTris-borate (1xTBE) buffer and the DNA fragment mixed with sample buffer was placed into selected well of the gel. DNA ladder was used to estimate the molecular weight of the fragment. Once the separation was complete it was viewed under a fluorescent lamp and visualized using ethidium bromide present in the gel. Upon successful identification of the V5 tag DNA, which is approximately 100 bp long, it was isolated using the RecoChip membrane. The membrane was placed into the gel in front of the desired band, then the electricity was turned on allowing the molecule to attach to it. The chip was later placed in a centrifuge and all of the bound plasmid or DNA on the chip was spun down, thus isolating the V5 tag.

9.2.4 Cloning of V5 tag into pZeoSV2(-)

Once the V5 tag was prepared and isolated it was further cloned into the pZeoSV2(-). The plasmid was first digested by previously mentioned restriction endonucleases s *XhoI* and *NotI*. Upon completion of digestion of the plasmid, the plasmid was separated on 0.7% agarose gel using gel electrophoresis method. Once successfully identifying the linearized plasmid, it was isolated using the RecoChip membrane (refer to the previous paragraph in section 8.2.3, on isolation of DNA from the agar gel).

The next step was the ligation of the isolated linearized plasmid with the V5 tag. During optimization different ratios of plasmid and insert have been tested, these include 1:2, 1:3, 1:5, 1:6, 1:9, 1:10 and 1:20. In ligation reaction the T4 DNA Ligase (along with T4 DNA Ligase

buffer) was used to clone V5 tag into the linearized plasmid, through the formation of phosphodiester bonds between the 3' hydroxyl end and a 5' hydroxyl end of DNA strands. Once the linearized plasmid and the V5 tag DNA optimization ratios have been chosen, they were mixed and heated at 55°C for 2 minutes and rapidly cooled on ice. After which T4 DNA Ligase (along with T4 DNA Ligase buffer) was added to the mixture and incubated at room temperature for 20 minutes to complete the ligation of the plasmid and V5 tag.

The ligation mixture was then transformed into *Escherichia coli* (*E.*coli) competent cells (strain dh5α) using the heat-shock method. First *E. coli* cells were mixed together with the ligation mixture, incubated on ice for 30 min and then heat shocked (42°C for 90 seconds). The heat shock forms pores in the cell membrane of the bacteria enabling the plasmid enter the cell. 1 mL of SOC (Super Optimal broth with Catabolite repression) medium was added to this mixture and incubated for an hour at 37°C/220 rpm. Once the transformation was complete the suspension was placed on the agar dish containing zeocin thus allowing only the transformed cells to grow. The dish was incubated at 37°C overnight. The next day most developed colonies were selected, re-picked onto a new zeocin agar dish and cultivated in the incubator at 37°C overnight. Simultaneously the cells were placed into the test tube with preheated zeocin LB (Lysogeny Broth) medium and allowed to grow at 37°C/220 rpm overnight. These colonies of bacteria multiply in the medium and with their multiplication also increased the concentration of the plasmid. The next day the bacterial cells were spun down and the pellet was used to harvest this highly copied DNA plasmid using the Plasmid DNA Purification kit obtained from Macherey-Nagel (Duren, Germany).

Once the purified plasmid was isolated from the bacteria the insertion of the V5 tag was verified by restriction analysis using either *BsaWI* or combination of *AgeI* and *BpmI* restriction endonucleases. Length of resulting fragments was visualized on the 0.7% agarose gel indicating whether the cloning was successful (the two different restriction analysis with resulting fragment lengths are seen in table 5).

Table 5: Restriction endonucleases used to digest pZSV5 and resulting fragment lengths

Restriction endonucleases	Resulting fragment length
AgeI + BpmI	1178, 2426
BsaWI	147, 1511, 1856

9.2.5 Cloning of V5 tag into pLNCX2

The V5 tag DNA used to clone into pLNCX2 retroviral plasmid was the same one used to clone into pZeoSV2(-) plasmid. The plasmid was first digested using restriction endonucleases *XhoI* and *NotI*, after which the plasmid was separated on the 0.7% agarose gel using the gel electrophoresis method. Upon successful identification of the linearized plasmid, it was isolated using the RecoChip membrane (refer to section 8.2.3, on isolation of DNA from the agar gel using RecoChip membrane).

The next step was the ligation of the linearized plasmid with the V5 tag. The optimization ratio of linearized plasmid and the insert which was tested was 1:3. To the ligation mixture the T4 DNA Ligase (along with T4 DNA Ligase buffer) was added to clone V5 tag into the linearized plasmid. The whole process was carried out by mixing the linearized plasmid and the V5 tag DNA together and heating the mixture at 55°C for 2 minutes then rapidly cooling the mixture on ice. The next step was the addition of T4 DNA Ligase (along with T4 DNA Ligase buffer) which was incubated at room temperature for 20 minutes to complete the ligation of the plasmid and V5 tag.

The ligation mixture was then transformed into *E. coli* competent (strain dh5α) cells using the heat-shock method (refer to method 8.2.4). Once the transformation was complete the suspension was placed on the agar dish containing neomycin thus allowing only the successfully transformed cells to grow. The dish was incubated at 37°C overnight. The next day most developed colonies were selected, re-picked onto a new neomycin agar dish and cultivated in the incubator at 37°C overnight. Simultaneously the cells were placed into the test tube with preheated neomycin LB medium containing neomycin and allowed to grow at 37°C/220 rpm overnight. The next day bacterial cells were spun down and the pellets were used to harvest the highly copied DNA plasmid using the Plasmid DNA Purification kit obtained from Macherey-Nagel (Duren, Germany).

Once the purified plasmid was isolated from the bacteria the insertion of the V5 tag was verified by restriction analysis using either *EcoRI* or *SpeI* restriction endonucleases. Length of resulting fragments was visualized on the 0.7% agarose gel indicating whether the cloning was successful (two different restriction analysis with resulting fragment lengths are seen in table 6).

Table 6: Restriction endonucleases used to digest pLNCXV5 and resulting fragment lengths

Restriction endonucleases	Resulting fragment length
EcoRI	1551, 1613, 3038
SpeI	2208, 3994

9.2.6 Amplification of BCRP cDNA

To amplify the cDNA of BCRP transporter using PCR one forward (hABCG2 v5f) and two different reverse primers were employed (hABCG2 v5r or hABCG2 v5r 2) (Figure 13).

Figure 13: Forward and Reverse primer sequences

The use of hABCG2 v5r reverse primer lead to amplification of the whole BCRP sequence, unlike the employment of hABCG2 v5r 2 produced BCRP sequence which is of 24 nucleotides shorter. Phusion polymerase (Finnzymes, Thermo Fisher Scientific, Vantaa, Finland) was employed for cDNA amplification in iQ Thermal Cycler (Bio-Rad, Hercules, California, USA) under the following conditions: 1 mM $MgCl_2$, 0.2 mM dNTP, 0.01 U/µL polymerase and 0.3 µM of each primer. To optimize the annealing step of the reaction temperature gradient was applied (50°-60°C). The resulting profile was as follows: 98°C for 30 seconds, 50 to 62 repeats of cycle consisting of 98°C for 7 seconds, 50°-62°C for 15 seconds and 72°C for 60 seconds.

Resulting PCR products were separated on 0.7 % agarose gel and visualized under UV light. The best amplified sample was selected and isolated from the gel using RecoChip membranes and prepared for cloning into either the pZSV5 or pLNCXV5 plasmids.

10. Results:

10.1.1 Preparation of V5 tag employing four oligonucleotides

The first part of the experiment involved preparing the V5 tag sequence using hybridization and further ligation of short sequences of oligonucleotides (V5N1, V5N1r, V5N2 and V5N2r). To get the desired sequence, different temperature profiles of hybridization were used. The first profile tested was: mixtures A (V5N1+V5N1r) and B (V5N2+V5N2r) were incubated at 80°C for 2 minutes. The reaction mixture was loaded on 2.5 % agarose gel and resulting V5 tag was visualised using gel electrophoresis method under UV light. As it can be seen from the photo of the gel (Figure 14) no clear band of the V5 tag was observed. Moreover, lots of impurities forming a smear can be seen on the gel. This indicated that the hybridization and ligation of oligonucleotides did not occur completely and that the temperature profile used did not lead to proper formation of V5 tag.

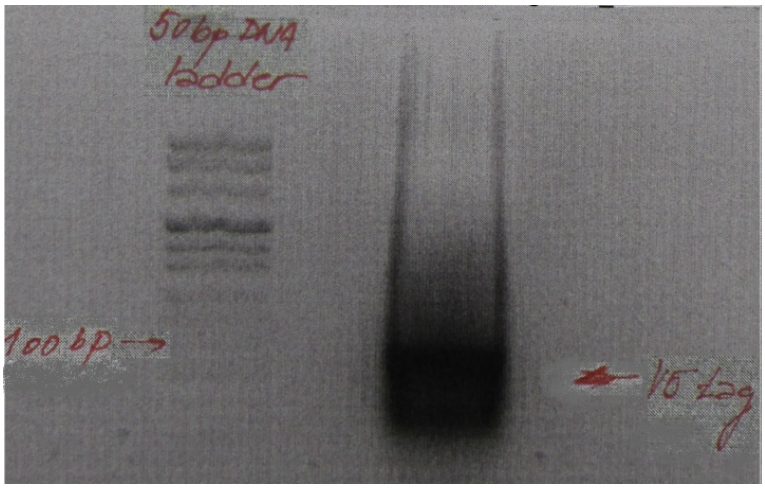


Figure 14: Photo of gel electrophoresis of V5 tag hybridization using the following temperature profile; mixtures A (V5N1+V5N1r) and B (V5N2+V5N2r) were incubated at 80°C for 2 minutes

Another tested temperature profile of hybridization was: mixtures A (V5N1+V5N1r) and B (V5N2+V5N2r) which were heated for 3 minutes at 90°C, followed by a 10 minutes decrease

in temperature to 70°C, then heated for 3 minutes at 70°C, followed by slow 10 minute temperature decrease to 37°C and finally were heated for 3 minutes at 37°C. When the reaction mixture was separated and visualized using gel electrophoresis method a clear band of molecular length around 100 bp was observed (refer to figure 15). This band corresponded with the expected position of the 96 bp long V5 tag, suggesting that the selected conditions of hybridization lead to proper assembly of oligonucleotides. The band was isolated from the gel via RecoChip membrane and used further in the cloning process.

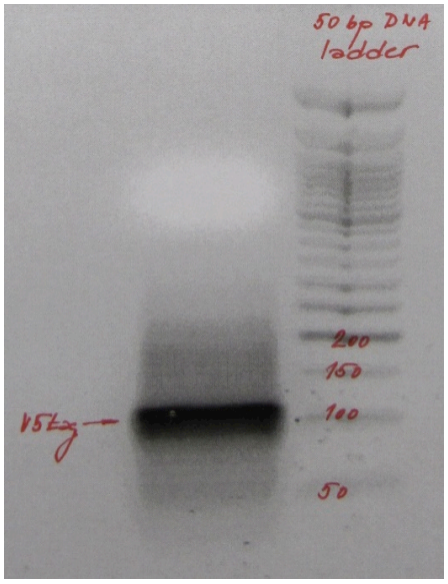


Figure 15: Photo of gel electrophoresis of V5 tag hybridization using the following temperature profile; mixtures A (V5N1+V5N1r) and B (V5N2+V5N2r) were heated for 3 minutes at 90°C, followed by a 10 minutes long decrease in temperature to 70°C, then heated for 3 minutes at 70°C, followed by slow 10 minute temperature decrease to 37°C and finally were heated for 3 minutes at 37°C.

In the third optimization process two different starting concentrations of oligonucleotides were tested, 100 µM and 10 µM. The temperature profile of hybridization was: mixture 1A (V5N1+V5N1r) and 1B (V5N2+V5N2r) were diluted ten times (100 µM) while the second mixture 2A (V5N1+V5N1r) and 2B (V5N2+V5N2r) were mixed using the original stock concentration (10 µM). Mixtures 1A, 1B, 2A and 2B were incubated at 95°C for 3 minutes and further incubated at room temperature over night. The resulting V5 tag was separated and visualised using gel electrophoresis method (Figure 16). In case of low starting oligonucleotides concentration no clear band corresponding with the expected V5 tag sequence was noted (Figure 16, lane 1A+1B). These results suggest that the concentration of oligonucleotides was not

sufficient to create the desired V5 tag. On the other hand, utilization of undiluted oligonucleotides resulted into a thick smeary band on the gel indication that the reaction mixture was overloaded (Figure 16, 2A+2B).

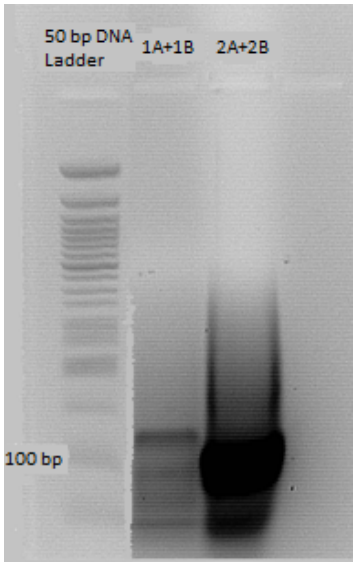


Figure 16: Photo of gel electrophoresis of V5 tag hybridization using the following temperature profiles: the mixture 1A (V5N1+V5N1r) and 1B (V5N2+V5N2r) were diluted ten times (1A+1B in the figure), while the second mixture 2A (V5N1+V5N1r) and 2B (V5N2+V5N2r) were mixed using the original stock concentration (2A+2B in the figure). Mixtures 1A, 1B, 2A and 2B were incubated at 95°C for 3 minutes and further incubated at room temperature over night

10.1.2 Preparation of V5 tag employing two oligonucleotides

Another approach of V5 tag assembly involved hybridizing two oligonucleotide sequences (V5_F and V5_R). The hybridization was tested using two different concentrations 100 µM and 10 µM, which occurred under following temperature profile: 10 times (100 µM) diluted mixture 3 (V5_F+V5_R) and undiluted (10 µM) mixture 4 (V5_F+ V5_R). Both samples were incubated at 95°C for 3 minutes, and then allowed to cool at room temperature over night. After the hybridization process was complete, resulting V5 tags was separated and visualised using gel electrophoresis method (refer to figure 17). Lane 3 in figure 17 demonstrates that low starting oligonucleotides concentrations did not produce the desired sequence of V5 tag. On the

contrary, when the oligonucleotides were mixed undiluted a clear band of an approximate length of 100 bp corresponding with V5 tag was observed (Figure 17, lane 4). As a result this band was isolated using the RecoChip membrane and used further in the experiment.

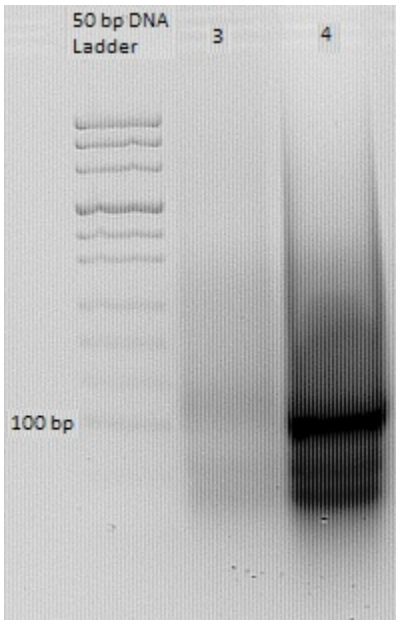


Figure 17: Photo of gel electrophoresis of V5 tag hybridization was tested using: 10-times (100 µM) diluted mixture 3 (V5_F+V5_R) in lane 3 and undiluted (10 µM) mixture 4 (V5_F+ V5_R) in lane 4. Both samples were incubated at 95°C for 3 minutes, and then allowed to cool at room temperature over night.

10.2 Cloning of V5 tag into pZeoSV2(-)

To prepare pZeoSV2(-) plasmid for cloning of the V5 tag into the plasmid, it was first digested using the restriction endonucleases which were *XhoI* and *NotI*. The whole reaction mixture was then loaded on the 0.7% agarose gel and visualized using the gel electrophoresis method. The length of 3500 bp was clearly identified when compared to the 1kb DNA ladder. The linearized plasmid was isolated using the RecoChip membrane, and further used in the experiment (refer to Figure 18).

Figure 18: Digestion of pZoeSV2(-) using *XhoI* and *NotI* restriction endonucleases. Band of the molecular length of 3500 bp was identified as the linearized pZeoSV2(-) plasmid.

For cloning of the V5 tag into the pZeoSV2(-) different optimization ratios of the linearized plasmid and insert were tested. The ratios of plasmid to insert which were tested are: 1:2, 1:3, 1:5, 1:6, 1:9, 1:10 and 1:20. The different ratios of linearized plasmid and V5 tag DNA were mixed and heated at 55°C for 2 minutes, and rapidly cooled on ice. After which they were ligated using T4 DNA Ligase (along with T4 DNA Ligase buffer) by incubating them at room temperature for 20 minutes. The ligated mixture was transformed into *E.coli* and allowed to grow on an agar dish containing zeocin thus allowing only the successfully transformed cells to grow. Four of the most developed colonies were selected from each ratio, with ratio 1:3 having the most developed colonies and also containing approximately 100 fully grown colonies on the plate to choose from. The colonies were further grown and the plasmids isolated (refer to method 8.2.4). The isolated plasmids were digested using *AgeI* and *BpmI* restriction endonucleases do determine if the V5 tag was ligated with pZeoSV2(-). The reaction mixture was loaded on 0.7 % agarose gel and the resulting plasmids were visualized, as seen in figures 19, 20 and 21. If the ligation of V5 tag with the plasmid was successful two clear bands should be observed at 1178 and 2426 bp on the agarose gel. All three figures give the same negative result, since all the ratios after visualization produced three bands which were all identical to the negative plasmid indicating that the ligation between pZeoSV2(-) plasmid and the V5 tag did not occur.

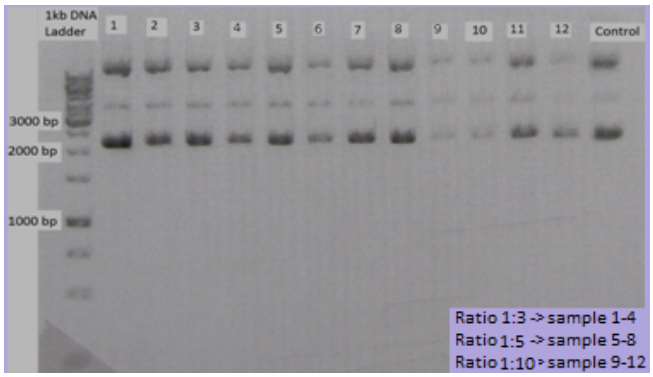


Figure 19: Restriction analysis of pZSV5. As a negative control original pZeoSV2(-) was used. Lanes 1-4: ratio1:3; lanes 5-8: ratio 1:5; and lanes 9-12: ratio 1:10

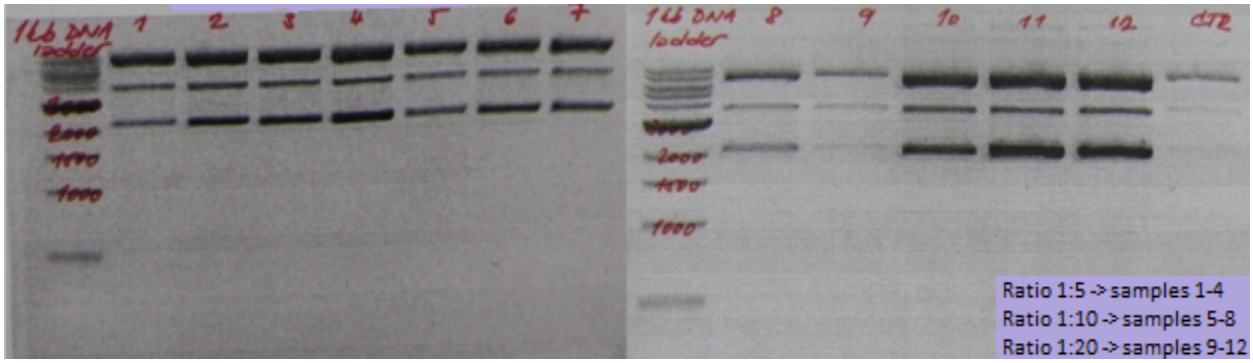


Figure 20: Restriction analysis of pZSV5. As a negative control original pZeoSV2(-) was used. Lanes 1-4: ratio1:5; lanes 5-8: ratio 1:10; and lanes 9-12: ratio 1:20

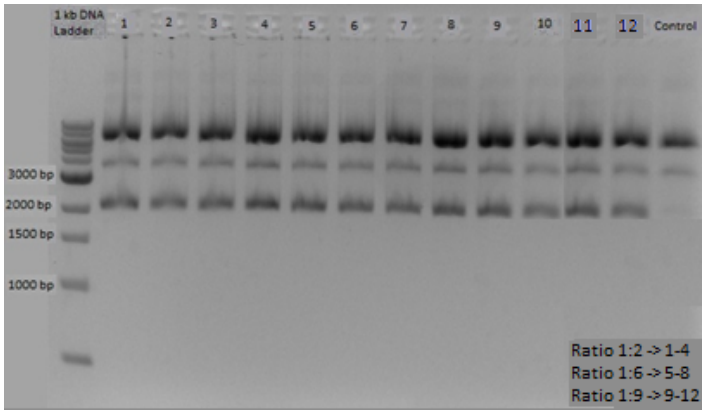


Figure 21: Restriction analysis of pZSV5. As a negative control original pZeoSV2(-) was used. Lanes 1-4: ratio1:2; lanes 5-8: ratio 1:6; and lanes 9-12: ratio 1:9

10.3 Cloning of V5 tag into pLNCX2

To prepare pLNCX2 plasmid for cloning of the V5 tag into the plasmid, it was first digested using the restriction endonucleases *XhoI* and *NotI*. The whole reaction mixture was further loaded on the 0.7% agarose gel and visualized using the gel electrophoresis method. The band length of 6100 bp was clearly identified when compared to the 1kb DNA ladder. The linearized plasmid was isolated using the RecoChip membrane, and further used in the experiment (refer to figure 22).

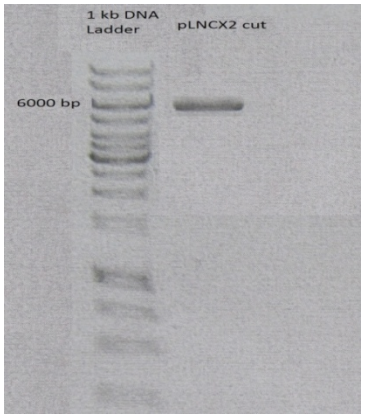


Figure 22: Digestion of pLNCX2 using *XhoI* and *NotI* restriction endonucleases. Band of the molecular length of 6100 bp was identified as the linearized pLNCX2 plasmid.

For cloning of the V5 tag into pLNCX2 plasmid the ration of the plasmid and insert used was 1:3. The reaction mixture was transformed into *E.coli* and allowed to grow on an agar dish containing neomycin thus allowing only the successfully transformed cells to grow. Ten of the most developed colonies were selected. The colonies were further grown and the plasmids isolated (refer to method 8.2.4). To verify that the cloning was correct the isolated pLNCXV5 plasmids were digested using *SpeI* or *EcoRI* restriction endonucleases.

When *EcoRI* was used nonspecific results were obtained (Figure 23). In most of the samples only two bands of approximately 1500 bp and 3000bp instead of three (1551 bp, 1613 bp, 3038 bp) were clearly visible under the UV light. On the contrary, the employment of *SpeI* restriction endonucleases revealed positive results in sample No. 1, 2, 3, 5, 6, 8, 9 and 10 (refer to Figure 24). The two bands observed at their specific lengths of 2200 bp and 4000 bp indicate

that in these samples the insertion of the V5 tag into pLNCX2 did occur. The most promising samples included 3, 6 and 8, were further isolated using the RecoChip membrane.

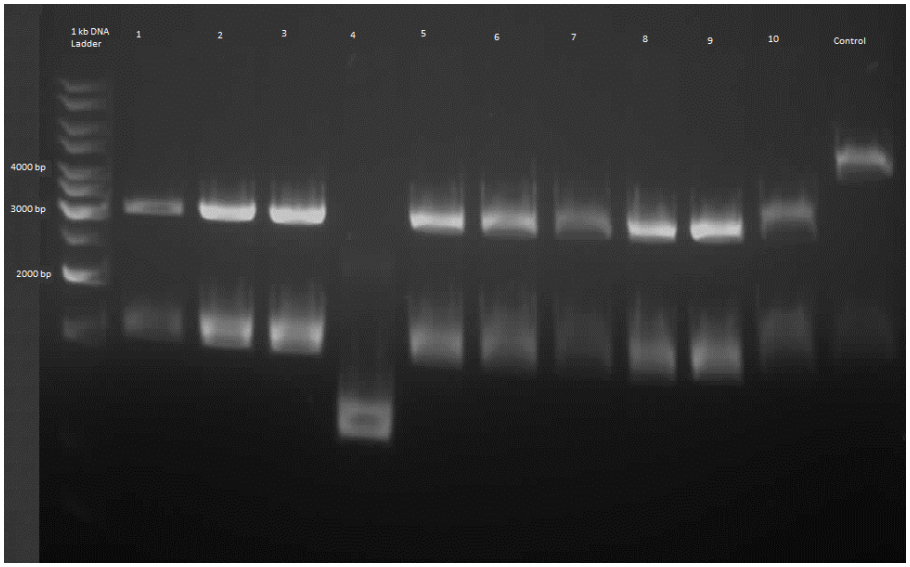


Figure 23: Restriction analysis of pLNCXV5 using *EcoRI* restriction endonucleases

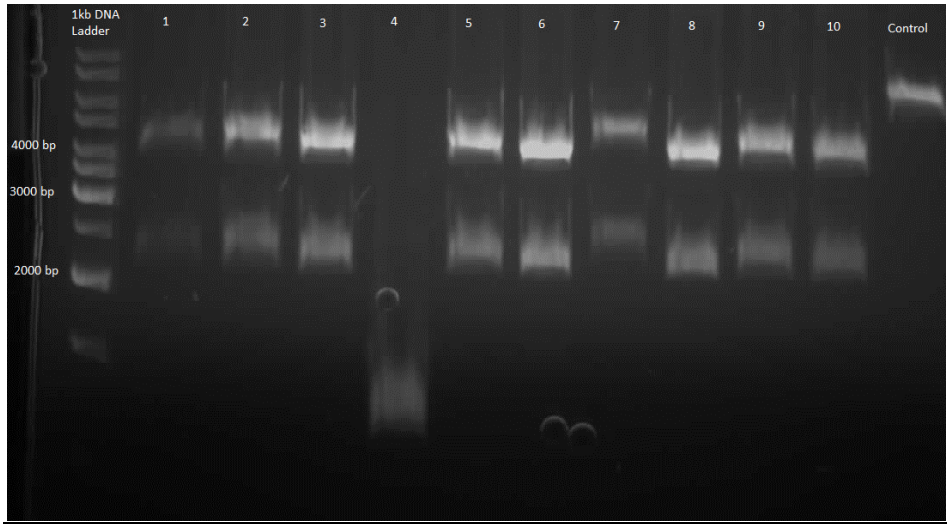


Figure 24: Restriction analysis of pLNCXV5 using *SpeI* restriction endonucleases. Bands of molecular length of 2200bp and 4000 bp were identified at their specific lengths indicating that the pLNCX2 plasmid was cloned with the V5 tag.

10.4 Amplification of BCRP cDNA

The BCRP cDNA insert was amplified from human liver cells using PCR method. Two different reverse primers were used leading to amplification of either the whole sequence of BCRP or coding sequence which was of 24 nucleotides shorter. However, no difference in amplification efficiency was observed between these two reverse primers. For annealing four different temperatures were analysed: 50°C, 53.4°C, 58.9°C and 62°C. All bands of the amplified BCRP cDNA when viewed on the 0.7% agarose gel resulted in specific lengths of approximately 2000 bp (Figure 25). Indicating that the BCRP cDNA was amplified correctly and that the best annealing temperature occurred at 62°C. The full sequence of BCRP cDNA at 62°C was isolated from the gel using the RecoChip membrane and prepared to be used further in the experiment.

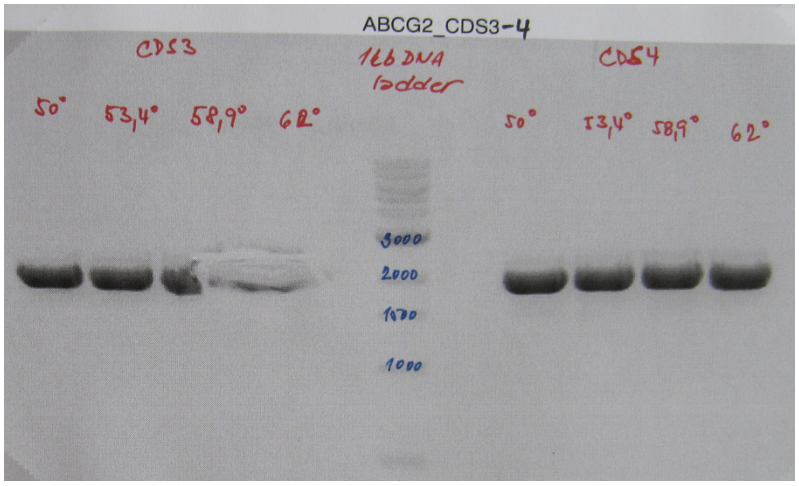


Figure 25: Photo of gel electrophoresis of amplification of BCRP cDNA using four different annealing temperatures, which were: 50°C, 53.4°C, 58.9°C and 62°C. CDS3 in this figure indicates that four samples of amplified BCRP were created using hABCG2 v5r reverse primer at temperatures specified previously, and lead to amplification of the whole BCRP sequence. CDS4 indicates that four samples of amplified BCRP were produced using of hABCG2 v5r 2 reverse primer at temperatures specified previously, as a result leading to production BCRP cDNA which was of 24 nucleotides shorter.

11. Discussion:

Goal of this study was to prepare a universal plasmid (pZSV5, pLNCXV5) which contains a short sequence of V5 tag. This plasmid enables further cloning of coding sequences of selected efflux transporters so that the V5 tag is localized on their 3'-end. Once constructed, the plasmid can be further used to transfect the desired cell line, thus developing an *in vitro* cell model. Since the transporter is produced as a fusion protein with the V5 tag localized on their C-end, the tag could be used as a universal marker of the transporter expression detectable by immunoanalytical methods (e.g. Western blot analysis).

The study begun with the development of the artificial V5 tag DNA. Different oligonucleotides, their starting concentrations and temperature profiles were employed in order to get the desired V5 tag by employing two different approaches for V5 tag assembly. One including the hybridization of two pairs of complementary oligonucleotides and their subsequent ligation, the other based on simple hybridization of the whole sequence from two complementary oligonucleotides. Both approaches proved to be suitable for V5 tag assembly. The success of hybridization and ligation in both was greatly affected by starting concentrations of oligonucleotides. Low concentrations resulted in an unclear band with lots of impurities forming a smear. In contrast, the use of high oligonucleotides concentrations resulted in clear band indicating successful preparation of the V5 tag. Furthermore, temperature profile of oligonucleotide hybridization of complimentary pairs were successful only when heated at three different temperatures; 90°C, 70°C and 37°C during the hybridization. On the contrary, hybridization of two complimentary oligonucleotide sequences forming the V5 tag sequence were successful at temperature profile of 95°C.

With a successful preparation of the V5 tag, the experiment was proceeded with the next step, which was the linearization of pZeoSV2(-) and pLNCX2 plasmids using the *XhoI* and *NotI* restriction endonucleases, preparing the plasmids to be ligated with the V5 tag. The attempt resulted in a successful identification and isolation of the linearized plasmids, which was followed by the ligation with the V5 tag. In order to optimize the ligation process.

Different ratios of the insert and plasmid were tested. Ratio 1:3 appeared to be the most optimal since it resulted in the most developed colonies, and also the individual colonies were evenly spaced out on the dish making the isolation possible without the chance of cross

contamination with the surrounding colonies. Nonetheless, all attempts made were unsuccessfully in creating the new pZSV5 plasmid. However, in case of pLNCXV5 the insertion of the V5 tag appeared to be successful. Then again the verification of the resulting plasmid was based only on the results from restriction analysis and further sequencing will be necessary to confirm the actual sequence of the pLNCXV5 plasmid. Only after this the plasmid could be used for cloning of BCRP and other efflux transporters and their subsequent expression in mammalian cells.

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